Elevated Fis Expression Enhances Recombinant Protein Production in *Escherichia coli*

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Abstract: A genetic strategy to enhance recombinant protein production is discussed. A small DNA bending protein, Fis, which has been shown to activate rRNA synthesis upon a nutrient upshift, was overexpressed in *E. coli* strain W3110 carrying vector pUCR1. Overexpression of Fis during exponential growth was shown to activate *rrn* promoters to different extents. A 5-fold improvement in chloramphenicol acetyltransferase (CAT) production in cultures with elevated Fis level was observed in shake-flask cultivations. A similar improvement in the culture performance was also observed during fed-batch fermentation; the specific CAT activity increased by more than 50% during the fed-batch phase for cultures with elevated Fis expression. In contrast, no increase in specific CAT activity was detected for cultures carrying pUCR2, expressing a frame-shift Fis mutant. Expression of Fis from a complementary vector, pKFIS, restored CAT production from W3110:pUCR2 to approximately the same level as cultures carrying pUCR1, indicating that the enhancement in CAT production was indeed Fis-dependent. The framework presented here suggests that differential activation in recombinant protein production may be achieved with differential Fis overexpression. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* **56:** 138–144, 1997.
Keywords: *rrn* promoter; rRNA synthesis; restricted growth; ribosome

INTRODUCTION

Escherichia coli is one of the most-studied organisms for recombinant protein synthesis. Typically, production of recombinant proteins in *E. coli* is severely curtailed under slow-growth conditions, such as during the production stage of fed-batch cultivation, due to the intense competition between normal cell function maintenance and recombinant protein production for the limited available metabolic machinery (San et al., 1994). Recent advents in genetic techniques have created new strategies for redesigning the protein synthesis machinery of *E. coli* to bypass some of these process bottlenecks. For example, the elimination of intracellular ppGpp has resulted in a five-fold increase in five recombinant protein synthesis during fed-batch fermentation (Dedhia et al., 1997). It is clear from this example that genetic modifications can be exploited to enhance protein synthesis for recombinant cells during restricted growth.

Fis is a 11.2 kDa, heat-stable DNA-binding protein in *E. coli* which has the ability to stimulate site-specific DNA inversion reactions by binding the protein to an enhancer sequence and bending the DNA (Johnson et al., 1986; Gille et al., 1991). In *E. coli,* the stable RNA operons have been shown to be *trans* activated by Fis (Nilsson et al., 1990). An upstream activator sequence (UAS) of these operons appears to be the target (Verbeek et al., 1990). All known ribosomal RNA (*rrn*) promoters have three consensus Fis-binding sites upstream of the P_1 promoter, and all have been shown to be *trans* activated by Fis (Ross et al., 1990). Mutations in Fis as well as deletion of Fis-binding sites cause a 2.5-fold decrease in *rrn* promoter activity (Condon et al., 1992). In vivo activation of the *rrn* promoters is especially essential in the presence of ppGpp, a nucleotide responsible for stringent control (Lazarus and Traver, 1993). On the basis of these results, it has been proposed that Fis serves an adaptive role, permitting high levels of rRNA transcription upon nutritional upshifts when RNA polymerase levels are depleted (Nilsson et al., 1992a). It appears that Fis bends the DNA and facilitates the binding of RNA polymerase (Gosink et al., 1993; Zacharias et al., 1992). However, recent findings suggest a cooperative interaction between Fis and RNA polymerase at the *rrn* promoter, which traps and recycles the limited RNA polymerase during the initial nutrient upshift (Bokal et al., 1995; Muskhelishvili et al., 1995).

Large fluctuations in Fis-dependent activation occur during a bacterial growth cycle after a nutritional upshift (Nilsson et al., 1992b). In early log phase, transcription of rRNA rises sharply and then declines (Ball et al., 1992). This is concomitant with a peak in the intracellular Fis concentration, suggesting that there is a correlation between changes of UAS-dependent activation and changes of Fis concentration. Expression of the Fis protein is not normally detected

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in stationary cultures. Adaptation to very fast growth requires this Fis-dependent activation; inactivation of the *fis* gene leads to reduced growth rate (Nilsson et al., 1992a). Many strains of *E. coli* deficient in Fis expression display increased lags upon subculturing, suggesting they have reduced ability to quickly respond to nutrient upshifts (Ball et al., 1992). Apparently, Fis-dependent activation is crucial to provide for high rates of rRNA synthesis required for rapid cell growth. Overexpression of Fis during the stationary phase results in higher cell death rates; cells that do not recover show prolonged lags before growth is resumed (Ball et al., 1992).

In this paper, we attempted to manipulate the protein synthesis machinery of slow-growing *E. coli* cultures to mimic that of exponentially-growing cultures by activating rRNA synthesis. The main objective was to enable cells to enhance recombinant protein synthesis under slow-growing conditions by manipulating the *trans* activation factor, Fis.

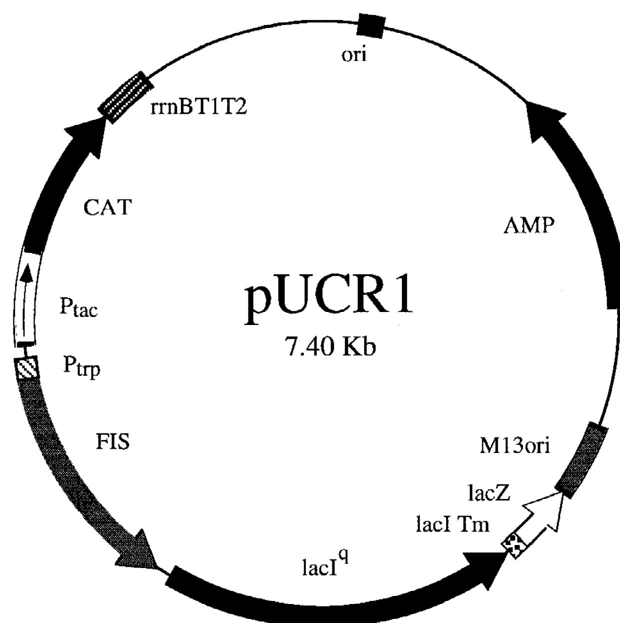

Figure 1. Plasmid map of pUCR1. Expression of Fis and CAT are regulated by the *trp* and *tac* promoter, respectively. A *lacI^q* gene is also included for tight regulation of the *tac* promoter.

MATERIALS AND METHODS

Strains and plasmids

E. coli strain W3110 (Bachman, 1987) was used for batch and fed-batch cultivations. Routine transformations were performed with *E. coli* DH5α [(*endA1 hsdR17 supE44 thi-1 recA1 gyrA* (*Nal^r*) *relA1* Δ(*lacIZYA-argF*)*U169 deoR* (ϕ*80dlac*Δ(*lacZ*)*M15*)]. *E. coli* strains W1485 ΔA, ΔC, ΔH (*rrn*A P1P2-CAT, *rrn*A P1P2-CAT, *rrn*H P1P2-CAT) (Condon et al., 1992) were used for *rrn* promoter analysis. Plasmid pRJ753 (Johnson et al., 1986), carrying the entire *fis* gene, was a gift from Dr. Reid Johnson. Vector pDR720, which carries the *trp* promoter, and the multi-cloning vector pSL1180 were obtained from Pharmacia. Plasmid pKC6, which expresses CAT under control of a *tac* promoter, has been described before (Chen et al., 1993). Plasmid pK184 (Bullock et al., 1987) is a low copy number, multi-cloning vector, which is compatible with vectors that carry the ColE1 origin.

Plasmids Construction and DNA Manipulations

A *Pvu*II-*Hind*III fragment, containing the entire *fis* gene, from plasmid pRJ753 was inserted into plasmid pSL1180 previously opened with *EcoR*V-*Hind*III to generate plasmid pSLFIS. To construct plasmid pTRPFIS, a *Sal*I-*Hind*III (Klenow filled in) *fis* fragment from pSLFIS was inserted into *Sal*I-*Bam*HI (Klenow filled in) digested pDR720. Finally, the *trp-fis* fusion from pTRPFIS was isolated as a 1 kb *Sph*I-*Sna*BI fragment, inserted into plasmid pKC6 digested with *Sph*I-*Xba*I (Klenow filled in). The resulting vector, pUCR1 (Fig. 1), expresses Fis and CAT under control of the *trp* and *tac* promoters, respectively. A control vector, pUCR2, carrying a frame-shift mutant of Fis was constructed by removing a *Bst*B I fragment, containing part of the *fis* gene, from pUCR1 to generate pUCR2. Plasmid pKFIS was constructed by subcloning a *Sph*I-*Sna*BI fragment, containing the *trp-fis* fusion, from pTRPFIS into a low copy number plasmid pK184, previously digested with *Sph*I-*Hinc*II.

All restriction endonucleases, modifying enzymes (T4 DNA polymerase, Klenow fragment, T4 DNA Ligase) and IPTG were purchased from New England BioLabs, Boehringer Mannheim Biochemicals, or Promega. Indoleacrylic acid (IAA) was obtained from Sigma Chemical (St. Louis, MO). All DNA manipulations were done according to standard methods (Sambrook et al., 1989).

Protein and CAT Assays

Cells were harvested by centrifugation and disrupted by sonication. The soluble fraction was used for protein and CAT analysis. Total protein concentration was determined using a Bio-Rad Protein Assay kit. CAT activity was assayed following the procedure described in Rodriguez and Tait (1983). Dilution of samples in TDTT buffer was required for samples with high activity. Kinetic measurements were performed with a Beckman DU 640 spectrophotometer in a 37°C temperature-controlled sample chamber.

Western Analysis

For SDS-PAGE analysis, cell lysates were boiled for 5 min in gel loading buffer (10% glycerol, 5% 2-mercaptoethanol, 3.3% SDS and 0.5 M Tris, pH 6.8) and then electrophoresed on a 12.5% polyacrylamide gel. For Western blot analysis, samples (10 μl) of 4× concentrated cells were combined with 5 μl of disruption buffer, and boiled for 5 minutes.

Samples were then electrophoresed through 12.5% SDS-PAGE gels (Laemmli, 1970) prior to staining or western blot analysis. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Western analysis was performed using a BioRad Immun-Blot GAR-AP kit (BioRad, Hercules, CA). Antibody prepared against FIS was generously donated by Dr. R. C. Johnson. Prestained low range protein markers (BioRad) were utilized for estimation of protein molecular weights.

Media and Growth Conditions

M9 medium (6.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 3 mg/L $CaCl_2$, 1 mL of 1M $MgSO_4.7H_2O$ and 4 mL of 20% Casamino acids) was used for both batch and fed-batch cultivations. For all experiments, media were supplemented with 0.2% glucose as the carbon source. Ampicillin or kanamycin was added at 50 mg/L or 10 mg/L, respectively, for selection. For the induction of CAT production, 0.5 m*M* IPTG was added unless otherwise described. Shake flask experiments were carried out at 300 rpm in a New Brunswick INNOVA 4000 incubator shaker at 37°C.

Fed-batch fermentations were carried out in a 5 L Bioflo 3000 (New Brunswick Scientific, Edison, NJ) bioreactor. The starting working volume was 4 L. The conditions of operation were: temperature, 37°C; agitation, 400 RPM; pH, 7.0; air flowrate, 2.0 L/min. pH was controlled by addition of NaOH and HCl. Foam was controlled by the addition of a 2% anti-foam A solution. The inoculum was grown in 250 mL of the same medium in a 1000 mL flask for approximately 16 hr. The amount of inoculum added to the bioreactor was adjusted to give a starting OD of 0.1. Feeding was initiated after the culture went through a second drop in growth rate (a second increase in DO from ~20% to 100%), indicating carbon limitation. A solution of 20% glucose and 10% casamino acids was fed to the bioreactor at a feed-rate of 4 ml/hr. Ampicillin was added continuously at a rate of 50 mg/hr to maintain selection pressure throughout the feed-batch fermentation.

RESULTS

Effect of Elevated Fis Level on rRNA Promoters

Although previous reports have indicated a definite link between Fis and rRNA expression (Zhang and Bremer, 1996; Ross et al., 1990), it is not clear whether overexpression of Fis during active growth conditions would result in a significant activation of the *rrn* promoters. The feasibility of Fis-activated rRNA expression was investigated by transforming the plasmid pTRPFIS into a set of *E. coli* strains (W1485 ΔA, ΔC, ΔH), containing a chromosomal integration of the upstream control region plus the promoters of the three *rrn* operons fused to a promoterless *cat* gene. Transcription from each *rrn* promoter was compared with the corresponding control strains (transformed with vector pDR720 without a *fis* insert), without Fis overproduction, in order to assess the effect of Fis-dependent *trans*-activation. As reported previously, the inherent promoter strength of each *rrn* promoter is directly reflected from the CAT activity measured (Condon et al., 1992). The average results from three shake-flask experiments are depicted in Figure 2. As can be seen, CAT expression from each *rrn* promoter was enhanced to varying degrees in the presence of elevated Fis expression. Moreover, addition of IAA alone or the presence of plasmid pDR720 alone had very little impact on *rrn* promoter activities. These results suggest that rRNA expression may be enhanced to a similar extent by the overexpression of Fis.

The extent of Fis expression was measured by Western blot analysis. Apparently, expression from the *trp* promoter is fairly leaky under our experimental conditions, as a fair amount of Fis was visible on the blot even without IAA induction (Figure 3, Lane 1). Addition of IAA resulted in only a small increase in Fis expression.

Effect of Fis Overexpression on CAT Production

An immediate objective is to study the effect of Fis-activated rRNA synthesis on recombinant protein synthesis. Even though elevated Fis levels result in elevated expression from the *rrn* promoters, it is not clear what Fis level is needed to achieve a significant improvement in recombinant protein synthesis. An optimal Fis level may exist that will significantly improve protein synthesis without a profound effect on cell viability. *E. coli* strains W3110 carrying pUCR1 and pUCR2 were used in these initial shake-flask experiments. Cells were grown in M9 medium until an OD of 0.3 before the addition of IPTG. In one of the flasks, 2.5 μg/mL of IAA was subsequently added to the cultures one hour after IPTG addition. The resulting profiles for cell growth and CAT production are shown in Figure 4. Independent of IAA induction, cells overexpressing Fis produced at least five-fold more recombinant CAT than the control. However, there was a noticeable decline in CAT activity as the cultures shifted into the slow-growing regime. Again, because of the nature of the *trp* promoter, expression was too leaky to obtain a good range of Fis overexpression (data not shown). However, a significant benefit in CAT production was still obtained.

Overexpression of Fis did not appear to impair cell growth nor viability significantly under our shake-flask conditions. Even though cell growth was reduced initially after IAA induction, there was only small deviations in the final cell density between cultures with and without Fis overexpression. Since overexpression of Fis under starvation condition is apparently lethal (Ball et al., 1992), it is important to investigate whether this strategy can be extended into fed-batch fermentation without significant losses in viability.

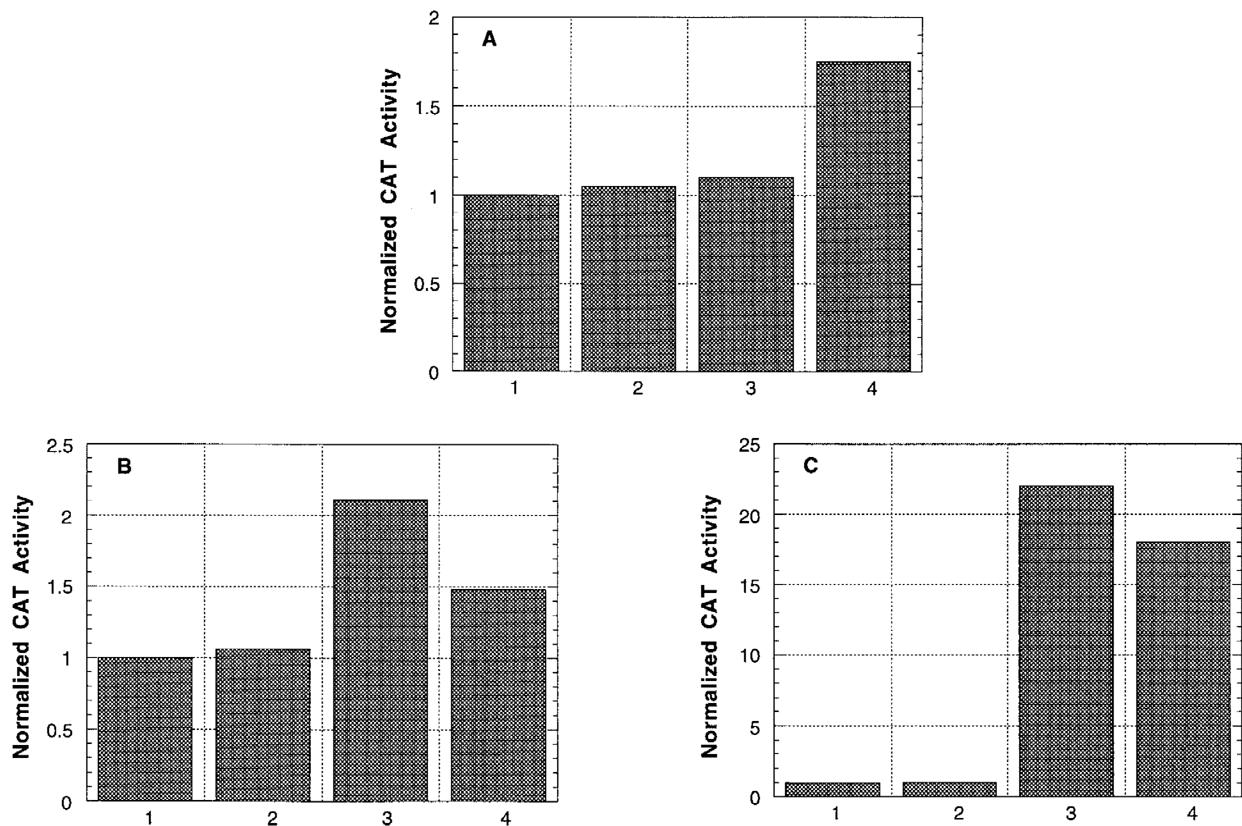

Figure 2. Activation of *rrn* promoters by Fis. A) CAT expression from *rrn*A promoter. B) Cat expression from *rrn*C promoter. C) CAT expression from *rrn*H promoter. Cells were grown in shake-flasks to an OD of 0.2 before the addition of IAA. Samples were collected 3 h after IAA addition. 1: no vector; 2: pDR720 (1.25 mg/μL IAA); 3: pTRPFIS (no IAA); 4: pTRPFIS (1.25 μg/mL IAA).

Enhancement of CAT Production during Fed-Batch Fermentation

Conceptually, elevated Fis expression should enable recombinant protein production to be extended far beyond the exponential growth phase into normally less productive regimens such as the stationary phase or slow-growing fed-batch phase. To explore this feasibility, fed-batch fermentations were conducted under controlled conditions. Cells were grown to an OD of 0.3 before induction with IPTG. Feeding was initiated after the culture went through a second increase in the DO from ~20% to 100%, indicating carbon limitation. As observed before in shake-flask cultures, CAT production was increased by about 5 fold in W3110:pUCR1 (Figure 5) during exponential growth. No increase in the specific CAT activity was detected in W3110:pUCR2 during the extended feeding phase, even though the total CAT activity was increased. On the other hand, a noticeable increase in both the total and specific CAT activity was observed in W3110:pUCR1. Particularly, the specific CAT activity increased by 50% in W3110:pUCR1, and did not decline throughout the whole period of feeding.

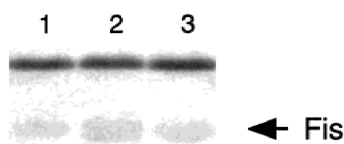

Figure 3. Effect of IAA induction on Fis expression. Western blot analysis of total protein from W1485 ΔA. Samples were collected as described in Figure 2. Lanes 1 and 3 represent cells carrying vector pTRPFIS with 0 μg/mL, 1.25 μg/mL, and 2.5 μg/mL IAA added, respectively. Arrow indicates the Fis protein band.

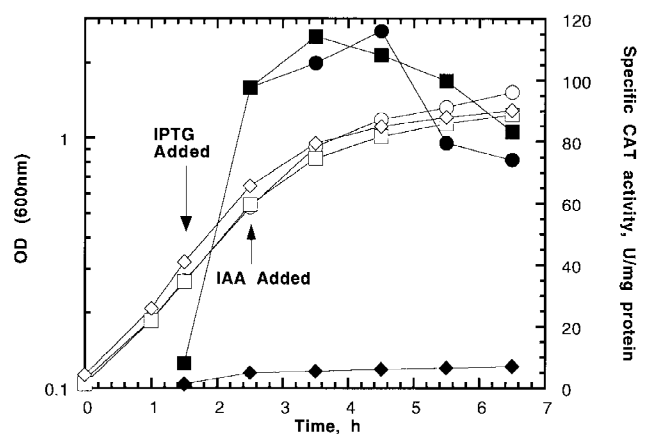

Figure 4. Cell growth (Open symbol) and CAT production (Closed symbol) from strain W3110 carrying vectors pUCR1 [0 mg/mL IAA (○) and 2.5 mg/mL IAA (□)] and pUCR2 (◇), respectively.

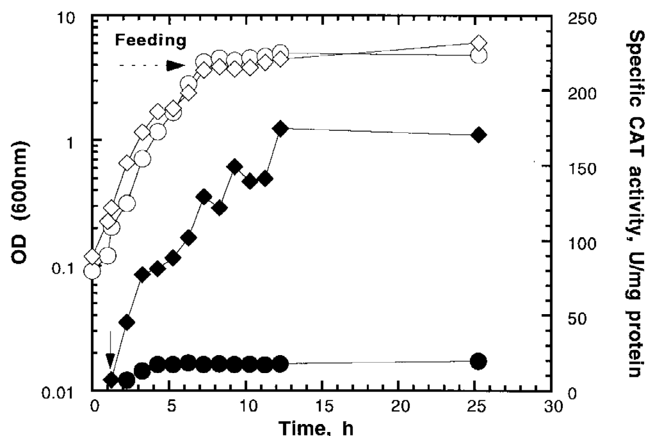

Figure 5. Comparison of cell growth (open symbol) and CAT production (Closed symbol) from strain W3110 carrying vector pUCR1 (◇) and pUCR2 (○) during fed-batch fermentation. IPTG addition is indicated by the arrow. The conditions of operation were temperature, 37°C; agitation, 400 RPM; pH, 7.0; initial air flow-rate, 2.0 L/min. Feeding was initiated after the culture went through a second increase in DO from 20 to 100%, indicating carbon limitation. A solution of 20% glucose and 10% casamino acids was fed the bioreactor at a feed-rate of 4 ml/hr. Ampicillin was added continuously at a rate of 50 mg/hr to maintain selection pressure throughout the feed-batch fermentation.

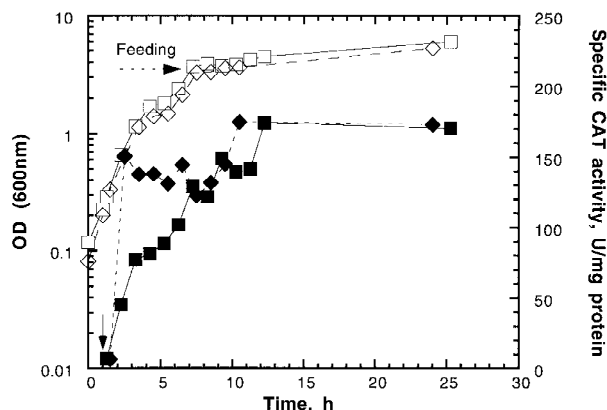

Figure 6. Complementation of CAT production in strain W3110:pUCR2 by vector pKFIS. Cell growth (Open Symbol) and CAT production (Closed Symbol) from strain W3110 carrying vector pUCR1 (□) and pUCR2 + pKFIS (◇) are shown. Fermentation conditions were the same as described in Figure 5 except kanamycin was also added to a concentration of 50 mg/L as a selection for plasmid pKFIS.

The final specific CAT activity from W3110:pUCR1 was about ten-fold higher than that from W3110:pUCR2. These results provide the first conclusive evidence of enhancing fed-batch phase recombinant protein synthesis using this genetically oriented strategy.

Again, cell growth and viability were not affected to a great extent in cells overexpressing Fis, especially during the fed-batch phase. The final culture density was approximately 10% lower for W3110:pUCR1. Attempts to highly induce Fis expression with up to 5 μg/mL IAA did not produce any significant difference in the final cell density or CAT expression (data not shown). An initial decline in CAT production and cell growth was observed. However, production and growth resumed and returned to the normal level shortly thereafter.

To demonstrate that the difference in CAT production between W3110:pUCR1 and W3110:pUCR2 is indeed a result of Fis deficiency, a low copy number, complementary vector, pKFIS, carrying a *trp-fis* fusion, was introduced into W3110:pUCR2. The resulting cultures were grown in the fermentor under conditions described above. As shown in Figure 6, CAT production was much higher than cultures carrying pUCR2 alone. Again, CAT activity increased by approximately 50% during the fed-batch phase. CAT production at the end of the fermentation was in line with that of cultures carrying pUCR1. Clearly, expression of Fis from a low copy number vector is sufficient to restore the high rate of CAT production as in cells carrying pUCR1. More importantly, this demonstrates that the difference in CAT production between cells carrying pUCR1 and pUCR2 is indeed Fis dependent.

DISCUSSION

Expression of recombinant proteins is, to a large extent, highly dependent on the physiological state of *E. coli.* Faster growing cells typically result in higher recombinant protein expression as demonstrated from the enhancement in VHb-expressing *E. coli* (Khosla et al., 1990). In some instances, it is possible to engineer the cellular machinery of slow-growing cultures to mimic the physiological state of a fast-growing culture. For example, recombinant protein production has been shown to increase in ppGpp-deficient (Dedhia et al., 1996) and KatF-deficient (Chou et al., 1996) *E. coli;* both products are responsible for the starvation response. Thus, it appears that certain distinct signals for slow-growing cultures can be manipulated to reverse their adverse effects.

Similarly, there are certain signals that are specifically turned on, enabling cells to rapidly resume growth upon nutrient upshift. A small protein, Fis, has been shown to be responsible in rapidly increasing ribosome concentration upon a nutrient upshift (Ball et al., 1992). Fis is transiently expressed for 1–2 hr. after a nutrient upshift, and mid-exponential or stationary cultures normally contain only very small quantities of Fis (Ball et al., 1992). We argue that overexpression of Fis under those conditions might also enable an increased expression from the *rrn* promoter. Indeed, expression from the *rrn*A, *rrn*C, and *rrn*H promoters was all increased under Fis overexpression, even though to different extents. In some cases, the improvement was as great as 20-fold. This difference in *rrn* promoter strength is greater than those observed between Fis$^+$ and Fis$^-$ cultures (Condon et al., 1992). This difference could be due to the fact that a much larger amount of Fis protein was expressed from our vector as compared to normal Fis expression on a single chromosomal copy. Whether or not this difference in Fis concentration is the determining factor in our observed differences in *rrn* promoter activity is still unclear. How-

ever, one might be tempted to suggest that differential *rrn* promoter activity can be achieved by differential Fis overexpression.

No one to date has reported how Fis overexpression might affect recombinant protein synthesis. Even though we have shown that *rrn* promoter activity was greatly enhanced, previous reports have shown very little impact on ribosome activity between Fis^+ and Fis^- cultures (Zhang and Bremer, 1996). However, overexpression of Fis during exponential growth may produce an entirely different set of responses. CAT production was greatly increased in cultures overexpressing Fis as compared to a control strain without Fis overexpression. The most significant improvement was observed in early exponential cultures. This is in agreement with other reports, indicating the importance of Fis in influencing ribosome synthesis during rapid cell growth (Zhang and Bremer, 1996). It should be noted that the specific CAT activity gradually declined upon the onset of stationary growth in shake flask cultures. It may be possible that Fis expression was also partially turned off, therefore, leading to the observed decline. Alternatively, overexpression of Fis during stationary growth may be lethal to the overall synthesis machinery (such as a decrease in transcription and translation or an increase in proteolysis) as suggested previously (Ball et al., 1992).

Similarly, elevated Fis expression also resulted in enhanced CAT synthesis during the fed-batch phase, although the increase was not as drastic as that observed during exponential growth. This observation is not surprising, as previous reports have also demonstrated the increasing importance of Fis on cell growth and rRNA synthesis with increasing growth rates (Zhang and Bremer, 1996). In contrast to W3110:pUCR2, CAT activity continued to increase during the fed-batch phase for W3110:pUCR1 even though the growth rate was relatively low. It is likely that, in the presence of continuous slow growth, Fis-dependent activation of recombinant protein synthesis can be sustained. Such a response is precisely what is needed in a wide variety of bioprocessing applications.

By complementing cells carrying pUCR2 with plasmid pKFIS, CAT production was restored back to the approximate level of cells carrying pUCR1. Specifically, CAT activity was also increased by ~50% during the fed-batch phase. These results clearly demonstrate that Fis is indeed responsible for enhancing CAT production during both exponential and fed-batch growth phases. We suspect that a broad spectrum of enhancement in recombinant protein may be possible if Fis expression is varied differentially. It is easy to envision that even very minimal Fis overexpression may be sufficient to provide a noticeable enhancement in recombinant protein synthesis. Such a strategy is currently under investigation.

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